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**9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)**

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Human neural cell types derived from human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) have a strong potential for use as cell-based biosensors for environmental toxins. Here, we report the following accomplishments in developing a neural cell-based biosensor. (1) We have developed methods to differentiate hESC-derived neural progenitor cells to dopaminergic-like neurons, allowing us to create a more physiologically relevant, HTS-ready in vitro model for Parkinson's disease research. (2) We have translated this dopaminergic differentiation methodology to hiPSC-derived neural progenitor cells. (3) As result of our dopaminergic differentiation studies, we have generated a unique, first-of-its-kind human dopaminergic progenitor cell that we are developing for commercial release. (4) We have developed methods to differentiate hESC-derived neural progenitor cells into astrocytes. (5) We have translated this astrocyte differentiation methodology to hiPSC-derived neural progenitor cells. (6) We have continued development of a fluorescence based, high content imaging (HCI) assay for neurogenesis with the potential as either a single or multiplexed assay format to identify compounds with effects on proliferation, differentiation and neurite outgrowth

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425 River Road
Athens, GA 30605

# Final Cumulative Report
# Human Neural Cell-Based Biosensor

**Date: May 28, 2013**

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**Contract Number: N00014-11-C-0011, Amendment/Modification P00002**

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**Abstract**

Human neural cell types derived from human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) have a strong potential for use as cell-based biosensors for environmental toxins. Here, we report the following accomplishments in developing a neural cell-based biosensor. (1) We have developed methods to differentiate hESC-derived neural progenitor cells to dopaminergic-like neurons, allowing us to create a more physiologically relevant, HTS-ready in vitro model for Parkinson's disease research. (2) We have translated this dopaminergic differentiation methodology to hiPSC-derived neural progenitor cells. (3) As result of our dopaminergic differentiation studies, we have generated a unique, first-of-its-kind human dopaminergic progenitor cell that we are developing for commercial release. (4) We have developed methods to differentiate hESC-derived neural progenitor cells into astrocytes. (5) We have translated this astrocyte differentiation methodology to hiPSC-derived neural progenitor cells. (6) We have continued development of a fluorescence based, high content imaging (HCI) assay for neurogenesis with the potential as either a single or multiplexed assay format to identify compounds with effects on proliferation, differentiation and neurite outgrowth to delineate mechanism-of-action for unknown neurotoxicants. (7) We have begun development of an improved cellular model system and neuromuscular junction bioassay for the detection and counteraction of botulinum neurotoxin poisoning. (8) The progress we have made has led to other successful grant applications and new and better ArunA product line development.

**Scientific and Technical Objectives**

The purpose of this project is to develop in vitro, cell based biosensors for environmental toxins. By using ArunA's neural cell lines derived from both human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), our goal is to provide a source of renewable neural networks and a human neural cell based biosensor that is a more biologically relevant model of human physiology for assessing human neural toxicity. The overall objectives for this project are (1) to develop methods for accelerating directed neuronal and glial differentiation for improved neural network formation and (2) to develop cell-based assays as sensor elements to detect cellular responses to test compounds. Over the course of this project, we have narrowed our scope and modified our objectives from those in the original proposal.

**Approach**

Over the course of the project, we have taken a more focused two-pronged approach to the development of a human neural cell based biosensor. We have therefore concentrated our primary efforts on the directed differentiation of human embryonic stem cell (hESC) and human induced pluripotent stem cell (hiPSC) derived neural progenitor cells. Specifically we are developing methods for neural progenitor cell differentiation into functional dopaminergic neurons as well as astrocytes, a glial cell type that promotes neuronal survival and formation of active neural networks. Our second focus is to evaluate the response of active neural progenitor cell-derived neuronal networks to common environmental toxins using cell-based assays as sensor elements. For our cell-based assays, we are continuing the development of (1) a fluorescence-based, high content imaging (HCI) assay for neurogenesis to monitor the effects of known and unknown toxicants on important neurodevelopmental processes, such as neurite outgrowth, proliferation and differentiation in vitro, and (2) botulinum neurotoxin (BoNT) detection and function assays. The development of fluorescence based assays as sensor elements was introduced as a new objective in Year 2 due to technical difficulties with growing active neurons on MEAs.

## Accomplishments

### *Outline*

*(1) Directed differentiation of hESC- and hiPSC-derived neural progenitor cells into dopaminergic neurons*
*(2) Directed differentiation of hESC- and hiPSC-derived neural progenitor cells into astrocytes*
*(3) Directed differentiation of hESC-derived neural progenitor cells into neural crest cells*
*(4) Development of cell-based assays as sensor elements*
*(5) Translation of work progress made to successful grant applications - Label-free, adhesive signature-based microfluidic cell separation*

### *(1) Directed differentiation of hESC- and hiPSC-derived neural progenitor cells into dopaminergic neurons*

We have continued our development of methods to direct the differentiation of hESC-derived neural progenitor cells into dopaminergic neurons. The rationale for creating dopaminergic neurons is the lack of renewable, scalable, patient specific and physiologically relevant in vitro models for Parkinson's disease.

We have been adapting methods previously developed (Young, et al., 2010) to differentiate hESC-derived neural progenitor cells into dopaminergic neurons. We have systematically screened different cell culture formats and basal mediums, as well as growth and induction factor formulations to enhance both the speed and yield of dopaminergic differentiation methods. We now have an established method for achieving dopaminergic differentiation in 3 weeks that demonstrates repeatable results and reproducibility among personnel (**Figure 1**).

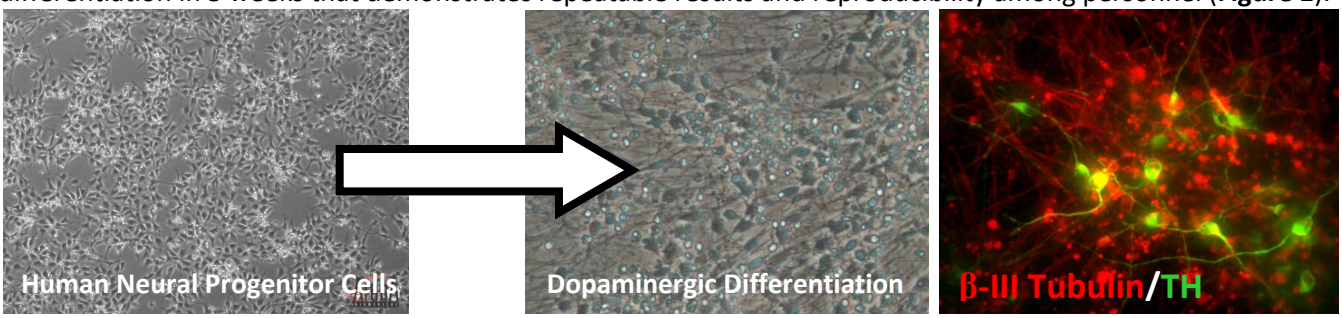


**Figure 1**. hESC-derived human neural progenitor cells (*left*) can be differentiated into dopaminergic neurons by 3 weeks (*right*). Differentiated dopaminergic neurons stain positive for mature neuronal marker β-III tubulin (*red*) and dopaminergic marker tyrosine hydroxylase (TH) (*green*).

As a result of our studies, we were able to identify and generate a unique, first-of-its-kind hESC-derived dopaminergic progenitor cell line primed for dopaminergic differentiation. This new dopaminergic progenitor cell line, branded DopaPro™, is >95% NURR1+ (**Figure 2**) and possesses many advantages including: 1) are robust and scalable for HTS format (96-,384-well) assays, 2) grow as adherent monolayers, and 3) possess a stable karyotype for multiple (>10) passages with a doubling time of ~36 hours.

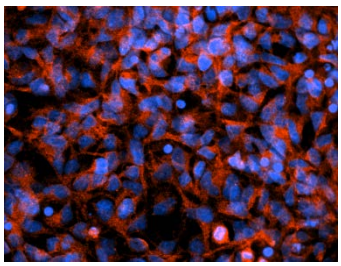

**Figure 2.** hESC-derived DopaPro™ cells stain >95% positive for NURR1, a marker indicative of dopaminergic progenitor cells.

As dopaminergic progenitor cells, DopaPro™ cells already primed for dopaminergic differentiation and the end user may skip over neural regionalization steps (including incubation with factors such as SHH) and proceed to

dopaminergic neuron maturation. We are now amplifying this line into working stocks for beta testing and for the commercial release of the DopaPro™ cells in a dopaminergic differentiation kit.

We have also completed preliminary HPLC studies evaluating our neural cultures for dopamine release with favorable results indicating dopamine biosynthesis and degradation, and further characterized our differentiated cultures for more extensive dopaminergic marker expression via immunocytochemistry and qPCR. We recently presented our results at the Annual Meeting of the Society for Neuroscience, October 13-17$^{th}$, 2012, in New Orleans, LA and at the Annual Meeting of the Society of Toxicology, March 10-14$^{th}$, 2013, in San Antonio, TX.

In preparation for the release of our dopaminergic differentiation kit, we have tested multiple different kit configurations and narrowed down the best options for kit configurations. We also continue to optimize and edit a differentiation protocol for customer use.

Although most of our work has focused on dopaminergic neuron differentiation of hESC-derived neural progenitors, our studies have also included the translation of our developed protocols to our hiPSC-derived neural progenitor cells. In our previous studies, we were able to generate a new hiPSC-derived neural progenitor cell line. Like their hESC-derived counterparts, these hiPSC-derived neural progenitor cells were derived, propagated, and maintained as adherent monolayers using serum-, feeder-free, defined medium. Since then, the hiPSC-derived neural progenitor cell line working stock has been amplified, characterized for karyotype and evaluated for the expression of neural progenitor cell phenotype markers and the ability to differentiate into pan neuronal cultures positive for markers of mature neurons. Furthermore, we have translated the dopaminergic differentiation process to hiPSC-derived neural progenitor cells with similar preliminary results showing positive protein expression of dopaminergic markers.

***(2) Directed differentiation of hESC- and hiPSC-derived neural progenitors into astrocytes***

The rationale for creating stem cell derived astrocytes is the lack of renewable, scalable and physiologically relevant in vitro models of neuron-glia interactions. By providing both neuronal and astrocytic cell types, a more complete and realistic model of human neural tissue can be created to reveal cellular neurotoxicological events.

Substantial progress has been made with establishing a robust astrocyte differentiation procedure for hESC-derived neural progenitor cells. For astrocyte differentiation, media conditions and components have been screened and refined, and gene and protein expression have been characterized at different time points (**Figure 3**). In all, we have developed a novel differentiation method that involves utilizing specific small molecules to reduce the differentiation time to produce astrocyte-like cells to 10 days, significantly faster than current protocols in the literature. Our findings were recently published in *Stem Cell Research* (see Publications).

Given the reproducibility of our astrocytic differentiation protocols, we have begun product development activities in preparation for the commercial release of our hESC-derived astrocytic cells line, branded hAstroPro™ cells. We have tested whether the astrocytic progenitor cells can be cryopreserved and thawed with acceptable levels of replating, and whether post thaw they can be co-cultured with neurons. To this end we have expanded and differentiated a production pilot run of hAstroPro cells, testing various starting cell lots and collecting cell samples at various differentiation time points for further gene expression analysis. We also compared different freezing media for their effect on the viability of hAstroPro™ cells post thaw. To enhance their utility in cell-based assays, various plating densities post thaw have been explored. Additional cell culture and media formulation experiments are also ongoing to enhance the maturity (GFAP expression) of the hAstroPro™ cells with extended culture. To date, we have successfully achieved high post thaw recovery rates.

In addition, post thaw astrocyte cultures were able to be successfully co-cultured with neurons. Functional network based electrophysiological studies have also been initiated to characterize network behavior of our neurons in these co-culture systems, and these studies remain in progress.

As a result of our studies we have also been able to make improvements to our neural basal medium to support the needs of both our pan neuronal cultures and astrocytes. We are currently in the process of testing our new medium formulation for commercial release. We have completed evaluating neural progenitor proliferation, mature neuronal differentiation and neural marker expression of cells cultured in this new medium. We are now in the process of characterizing and validating the new medium for astrocyte differentiation of both hESC- and hiPSC-derived neural progenitor cells and resulting glial marker expression.

Preliminary work on translating astrocyte differentiation protocols to hiPSC-derived neural progenitor cells has also been conducted, demonstrating that this cell line is amenable to astrocytic differentiation as well (**Figure 3**).

### *(3) Directed differentiation of hESC-derived neural progenitor cells into neural crest cells*

Separating and migrating from the neural tube during embryonic development and giving rise to a wide variety of cell types, neural crest cells have garnered increasing interest as new models of developmental neurotoxicity caused by environmental factors. Given the rising interest in neural crest cells as a means to create a human in vitro model to predict human health outcomes, we began preliminary work on differentiating ArunA's hESC-derived neural progenitor cells into neural crest cells following our own unique protocols. Cells were differentiated under different conditions with varying combinations of growth factors, media formulations, and plating densities, and resulting cultures were analyzed for neural crest marker protein and gene expression using immunocytochemistry and RT-PCR to identify those conditions most efficiently yielding a neural crest cell phenotype.

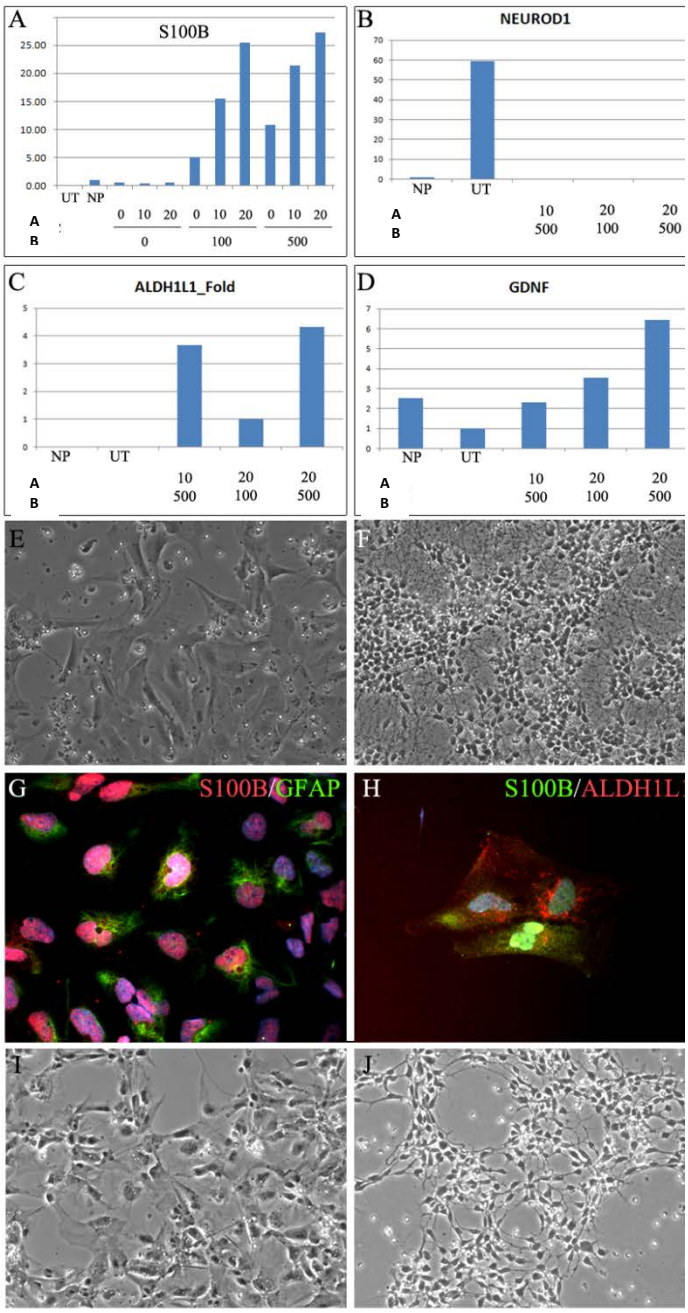


**Figure 3: Derivation of astrocytes from neural progenitor cells of hESC and hiPSC origin**. qPCR dose-dependent changes in expression of astrocyte marker S100B with combinations of Small Molecules A and B were observed upon differentiation (A). Up-regulation of neuronal marker NEUROD1 is seen after 5 days of neuronal differentiation (UT), while transcripts are undetectable after astrocytic differentiation (B). Expression of (C) ALDH1L1 and (D) higher GDNF expression were seen with combined Small Molecule A at 20nM and Small Molecule B at 500nM. Morphological differences between astrocytic and neuronal differentiation (E) and (F) respectively, after 15 days in culture. Expression of S100B (red) and GFAP (green) after 30 days of differentiation (G), and expression of S100B (green) and ALDH1L1 (red) (H). Astrocytic and neuronal differentiation morphology after 10 days (I and J respectively).

### *(4) Development of cell-based assays as sensor elements*

We continue to make substantial progress in developing cell based assays as sensor elements for environmental toxins. In particular we have focused on establishing a neurogenesis assay using a fluorescence-based, high content imaging (HCI) platform, as well as a botulinum neurotoxin assay. To focus on these assays, the cellular ATP, alamarBlue assay, ROS assay and cell migration assay have recently been put on hold.

**Fluorescence-based high content imaging (HCI) assay:** Previously, we had assessed neurotoxicants in a neurite outgrowth assay using the already differentiated, mixed human neuronal hN2™ cell line (Harrill, et al., 2010). In this project, we have expanded our studies to examine neurogenesis in our hESC-derived neural progenitor cells using a fluorescence-based ImageXpress® high content imaging (HCI) platform. Using this assay platform allows us to monitor human neural progenitor cell proliferation, as well as differentiation in terms of neurite outgrowth. This assay allowed for the detection of neurotoxicants, as well as positive and negative modulators of neural progenitor cell proliferation and neuronal differentiation, with output parameters including the number of neurites, length, branches, etc. per cell or per field. Those conditions that promoted mitosis and mitigated apoptosis, as well as those factors increasing the number of neurons and enhancing neurite outgrowth could be readily identified using the ImageXpress® platform. Also, we were able to make further progress with our already differentiated human neuronal hN2™ cells, by identifying among a test set of compounds which ones had potential cytotoxic effects and led to disintegration of neuronal networks. In continued HCI studies, we are currently expanding our test set of known inducers of apoptosis and mediators of neuronal toxicity to market our hNP1™ and hN2™ cell lines with high content imaging platforms as a screening tool for drug discovery and toxicology studies. Results from our studies have been presented in both oral presentation and poster format at the Society for Neuroscience Annual Meeting, November 12-16$^{th}$, 2011, Washington, D.C. and Society of Toxicology Annual Meeting, March 11-15$^{th}$, 2012, San Francisco, CA.

**Botulinum neurotoxin (BoNT) assay**: We have continued development of a human neuronal hN2™ cell based sensor for botulinum toxin (BoNT) detection through our sub contractor, Dr. Julie Coffield, to determine the lowest sensitivity of the cells to BoNT-A treatment resulting in cleavage of SNAP-25, the target for BoNT proteolytic activity. Thus far, BoNT has cleaved SNAP-25 in hN2™ cells at concentrations as low as 10pM. We are also working on establishing a faster means of generating mixed neuronal hN2™ cell cultures that have a higher percentage of motor neurons by using less expensive activators of motor neuron differentiation. Initially we have been using a mouse ESC as a model system to develop these faster differentiation protocols. Currently we have generated mESC-derived neuronal cultures with > 40% differentiation to motor neurons using puromorphine. These cells can excite mouse C2C12 muscle cells via neuromuscular junctions, and we are currently testing a quantifiable bioassay for neuromuscular junction function in conjunction with the University of Illinois. A functional and scalable bioassay for neuromuscular junctions is of significant interest to private and government research directed at counter measures for BoNT poisoning.

**High throughput automated patch clamp systems:** Development has resumed on the large scale screening of compounds for effects on ion channel activity through direct electrophysical measurements using an IonWorks Barracuda™ system. Using this high throughput automated patch clamp system, the electrophysiological and pharmacological properties of endogenous ion channels in hN2™ cells can be characterized by measuring ligand and voltage-gated ion channels in 384 parallel recording sites. Such a platform technology can accelerate drug discovery, as well as evaluate neurotoxicity of compounds that are ion channel targets. Results were presented at the Society for Neuroscience Annual Meeting, November 12-16, 2011, Washington, D.C.

### *(5) Label-free, adhesive signature-based microfluidic cell separation*

We received an award letter from NIH on a $700K grant to fund the development of a label-free microfluidic device for commercialization. The progress we have made in this present project contributed to a successful grant application and potentially new and better ArunA product lines in the future. Due to our work progress in this project, we were able to initiate a collaboration with Georgia Tech to establish the feasibility of using a novel microfluidic-based approach to efficiently isolate different cell populations based on their distinct 'adhesive signature' via controllable fluid forces. This label-free and non-enzymatic method is faster, simpler and higher throughput than current cell separation techniques. If effective across multiple hiPSC lines (both healthy and diseased) and multiple derivations of neural cell types, this novel adhesion-based, label-free microfluidic system will shorten and streamline the scaled-up production of enriched populations of hiPSC-derived neural rosettes, neural progenitor cells and mature neuronal and glial cell types for direct use in cell-based assays. Our work with Georgia Tech has been published in *Nature Methods* (see Publications). With our collaboration with Georgia Tech effectively underway, we are currently evaluating cell surface protein properties of our neural cell lines successfully generated under this contract in order to best optimize microfluidic cell separation methods.

**Conclusions**

In summary, we have established focused methodologies to direct the differentiation of hESC- and hiPSC-derived neural progenitor cells to dopaminergic neurons and astrocytes. Along the way we have been able to initiate the development of new neural products for commercial release. By differentiating our hESC- and hiPSC-derived neural progenitors to specific neural subtypes, we aim to increase the versatility of our neural cell-based biosensors by incorporating cell type specificity, as well as the ability to create disease-specific systems. Our work here also strongly suggests that our fluorescence-based HCI assay is a powerful platform allowing us to examine novel neurotoxicants for their capacity to perturb neural cellular processes.

**Significance**

Efficient identification and prioritization of chemicals potentially hazardous to humans is imperative to reduce significant health risks posed by environmental exposure. Recent publications advocate the use of human cell-based models to reduce the uncertainties seen with the use of non-human animal models to evaluate the human health effects of chemicals with unknown toxicological properties. In response to the call for human cell-based models, we are developing a human neural cell-based biosensor platform consisting of functional human neuronal and glial cell types from differentiated hESC- and hiPSC-derived neural progenitor cells. We believe the work progress described above will have significant impact beyond the scope of this project. These stem cell-derived neural cell types represent a renewable and more physiologically relevant source of neural cells for creating cell-based sensors to assess human neural toxicity. Utilizing these differentiated cell types in combination with cell-based assays, we have the potential to identify compounds with human toxicity more quickly and reliably and to delineate compound mechanism-of-action. The studies initiated here in this project have set the stage for ArunA to commercially provide researchers and institutions with specialized, even patient specific, first generation human cellular model systems for toxin detection.

**Publications (cumulative for contract period)**

*Refereed journal articles*

Majumder A, Dhara SK, Swetenburg R, Mithani M, Cao K, Medrzycki M, Fan Y, Stice SL. Inhibition of DNA methyltransferases and histone deacetylases induces astrocytic differentiation of neural progenitors. Stem Cell Res. 2013 Apr 2;11(1):574-586.

Singh A, Suri S, Lee T, Chilton JM, Cooke MT, Chen W, Fu J, Stice SL, Lu H, McDevitt TC, García AJ. Adhesion strength-based, label-free isolation of human pluripotent stem cells. Nat Methods. 2013 May;10(5):438-44.

Majumder A, Banerjee S, Harrill JA, Machacek DW, Mohamad O, Bacanamwo M, Mundy WR, Wei L, Dhara SK, Stice SL. Neurotrophic effects of leukemia inhibitory factor on neural cells derived from human embryonic stem cells. Stem Cells. 2012 Nov;30(11):2387-99.

West FD, Henderson WM, Yu P, Yang JY, Stice SL, Smith MA. Metabolomic response of human embryonic stem cell-derived germ-like cells after exposure to steroid hormones. Toxicol Sci. 2012 Sep;129(1):9-20.

Dodla MC, Young A, Venable A, Hasneen K, Rao RR, Machacek DW, Stice SL. Differing lectin binding profiles among human embryonic stem cells and derivatives aid in the isolation of neural progenitor cells. PLoS One. 2011;6(8):e23266.

Dhara SK, Majumder A, Dodla MC, Stice SL. Nonviral gene delivery in neural progenitors derived from human pluripotent stem cells. Methods Mol Biol. 2011;767:343-54.

Young A, Machacek DW, Dhara SK, Macleish PR, Benveniste M, Dodla MC, Sturkie CD, Stice SL. Ion channels and ionotropic receptors in human embryonic stem cell derived neural progenitors. Neuroscience. 2011 Sep 29;192:793-805.

Krishnamoorthy M, Gerwe BA, Scharer CD, Heimburg-Molinaro J, Gregory F, Nash RJ, Arumugham J, Usta SN, Eilertson CD, Stice SL, Nash RJ. GABRB3 gene expression increases upon ethanol exposure in human embryonic stem cells. J Recept Signal Transduct Res. 2011 Jun;31(3):206-13.

Gerwe BA, Angel PM, West FD, Hasneen K, Young A, Orlando R, Stice SL. Membrane proteomic signatures of karyotypically normal and abnormal human embryonic stem cell lines and derivatives. Proteomics. 2011 Jun;11(12):2515-27.

Callihan P, Mumaw J, Machacek DW, Stice SL, Hooks SB. Regulation of stem cell pluripotency and differentiation by G protein coupled receptors. Pharmacol Ther. 2011 Mar;129(3):290-306.

*Workshop/conference abstracts, presentations, posters, and papers*

Powe, A, et al. *Development of a Novel Assay Platform for Visual Assessment of hESC Derived Neural Progenitor Cell Migration in Response to Neurotoxicants***,** Society of Toxicology Annual Meeting 2011

Powe, A, et al. *Development of a novel high throughput screening/high content imaging amenable assay for cell proliferation and migration in human embryonic stem cell derived neural progenitors.* Society for Neuroscience Annual Meeting 2011.

Sirenko, O et al. *High Content Image Analysis of Neurogenesis Using Human Embryonic Stem Cell Derived Neural Cells.* Society of Toxicology Annual Meeting 2012.

Stice, Steven L. *Advancing the Science of Stem Cells in Toxicology*. Society of Toxicology Annual Meeting 2012.

Chilton, J.M. *Derivation of scalable, NURR1 expressing dopaminergic progenitor cells from human embryonic stem cells.* Society for Neuroscience Annual Meeting 2012.

Chilton, J.M*. Scalable, HTS/HCI-amenable and NURR1-expressing dopaminergic progenitor cells derived from human embryonic stem cells.* Society of Toxicology Annual Meeting 2013.

**Patent information (cumulative for contract period)**

**Technology transferred (cumulative for contract period)**

We continue to develop customers and collaboration throughout Government and industry. For example, we have an ongoing collaboration with EPA to develop developmental neurotoxicity assays. Recently we have developed further application in metabolomics with a separate branch at EPA in Athens. We continue to keep in contact with members of congress including our two US senators from Georgia, Chambliss and Isakson, who have both visited our labs in Athens. ArunA sales continue to grow in both industry and academia. Life Tech is a distributor of our cells and scientist from GSK, Novartis Merck have all purchased from ArunA.

**Awards/Honors received during contract period**

# Human Neural Cell Based Biosensors for Environmental Toxins

Steven L. Stice, ArunA Biomedical, Inc.

**Objective:**

- Develop human neural cell based biosensor using ArunA's neural cell lines derived from human embryonic stem cells (hESC) and human induced pluripotent stem cells (hiPSC)

**Approach:**

- Directed differentiation of neural progenitor cells using defined media formulations and small molecules into specific neural subtypes
- Neural subtypes are characterized by specific marker expression
- Development of HCI assays for neurite outgrowth

**Accomplishments:**

- Astrocyte differentiation
- Dopaminergic Differentiation
- Neurite outgrowth assays developed demonstrate utility in detecting neurotoxicity

**Impact/Transitions:**

- Publications submitted
- New neural product lines in development
- New grant funds awarded